Supplementary Information: Menge et al.

Materials and methods:

Primary Cells:

First-passage human MSCs and PECs were purchased from Lonza (Walkersville, Maryland). MSCs were found to have undetectable levels of major histocompatibility complex (MHC) class II and very low levels of MHC class I; intermediate levels of the cellular adhesion proteins ICAM; and high levels of CD105, CD29, and CD44(2;6). MSCs were cultured in (MSCGM) (Lonza). PECs were cultured in (EGM2) (Lonza, Walkersville, MD). Cells were used at passages 2–7. Murine C57Black6 MSCs were obtained from Gibco/Life Technologies (Grand Island, NY). Mouse MSC growth medium consists of D-MEM/F-12 medium with GlutaMAX-I, 10% MSC-Qualified FBS, and 5 µg/mL gentamycin reagent solution. All reagents were supplied by Gibco/Life Technologies (Grand Island, NY). Cells were passaged similar to human MSCs. U937 cells were obtained from ATCC and passaged in RPMI 10% FBS.

MSC-PEC Co-culture and MACs Cell Separation

PECs were co-cultured in contact with MSCs or with MSCs in transwells. The ratio of MSCs to PECs was 1:5. Cells were harvested by trypsinization after 24 hr. and sorted by magnetic cell sorting (MACS) CD31 beads (Miltenyi Biotec, Germany) as per manufacturer's instructions. The CD44 (negative)-population of (PECs) was used in subsequent assays of endothelial function. An aliquot of this population of cells was confirmed to be CD31+ by flow cytometry.

RNA Preparation and Quantitative RT-PCR

RNA from cells and tissue was collected using the mirVana isolation Kit (Ambion, Austin, TX). cDNA was synthesized from 0.1-1.0 ug of total RNA and TaqMan probes for human or mouse TIMPs and GAPDH (proprietary sequence) were used (Applied Biosystems Carlsbad, CA). Amplification was measured using quantitative RT-PCR system (Step One Plus-Applied

Biosystems Austin, TX). Relative mRNA expression is representative of differences in relative quantity (RQ) of each amplified gene, and was analyzed using a Student's t-test for 2 group comparisons.

Affymetrix Arrays and Biostastical Analysis

The Affymetrix Human Genome U133 Plus 2.0 GeneChip® (Santa Clara, CA) was used for gene expression profiling. Each of arrays contains 54,675 probe sets including 62 controls, representing 39,000 transcripts and ESTs. The raw data was quantified using Robust Multiarray Analysis (RMA) algorithm(*31*). To identify differentially expressed genes between two groups, two-sample t-test was utilized on probe set-by-probe set basis; the p-value for each probe set was computed based on the test statistic applied(*32*). Supervised two-way hierarchical clustering technique was utilized to illustrate findings. To produce two-way clustering heat map, the Pearson correlation was used for distance matrix calculation and the Ward's method was applied as linkage rule. The expression data processing and analyses were performed using R (version 2.10.0) and Bioconductor packages (http://www.bioconductor.org/). To better understand genes differentially expressed between PECs cultured alone or co-cultured with MSCs and then separated, we used clustering to group genes with appropriate p-values (FDR < 1%) into sets that exhibited similar expression patterns. Gene annotation enrichment analysis of clustered genes was performed using the NIH DAVID annotation tool (http://david.abcc.ncifcrf.gov).

Immunofluorescence of Endothelial Cell VE-Cadherin/β-catenin and Occludin

Endothelial cells were treated with rTIMP3 (1.0 µg/mL) or other treatment groups in EGM2 and maintained in culture for 1 hr. before addition of VEGF-A165. Cells fixed in 4% paraformaldehyde then were incubated with primary antibodies for VE-cadherin and or β-

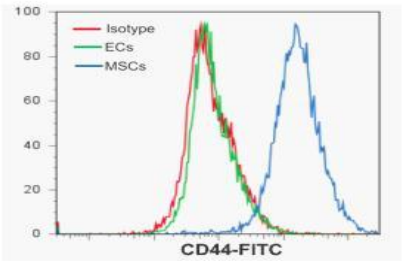
catenin (Cell Signaling Technology, Danvers, MA). Quantitation was conducted as described previously(*3*).

Immunohistochemistry of cortical inflammation post-TBI

Mice were sacrificed 3 days after injury and brains were quickly removed and snap frozen in -80°C isopentane. Coronal sections (10 mm) were cut on a cryostat, mounted on slides, postfixed for 20 min with -20°C methanol, and rehydrated in PBS. Sections were incubated in primary antibodies against inflammatory cell markers: NIMP-R14 (Abcam, Cambridge, MA) for neutrophils, Iba (Wako Richmond, VA) for microglia and GFAP (Sigma-Aldrich) for astrocytes, (1.0 μg/mL in PBS containing 2% goat serum and 0.1%Triton X-100) for 18 h at 4°C, washed in PBS, and then incubated with Alexa Fluor-conjugated species-specific secondary antibodies (Invitrogen) for 1 h at RT. Sections were cover slipped with Fluormount-G (Fisher Scientific), and imaged using an upright Olympus BX-51 microscope (Bio-Rad Hercules, CA) attached to a MagnaFire digital camera (Optronics Goleta, CA). Images were taken surrounding penumbra area of cortex on 4 brain sections containing the lesion core for each animal and up to 4 mice were included in each treatment group.

Supplementary Figure 1.

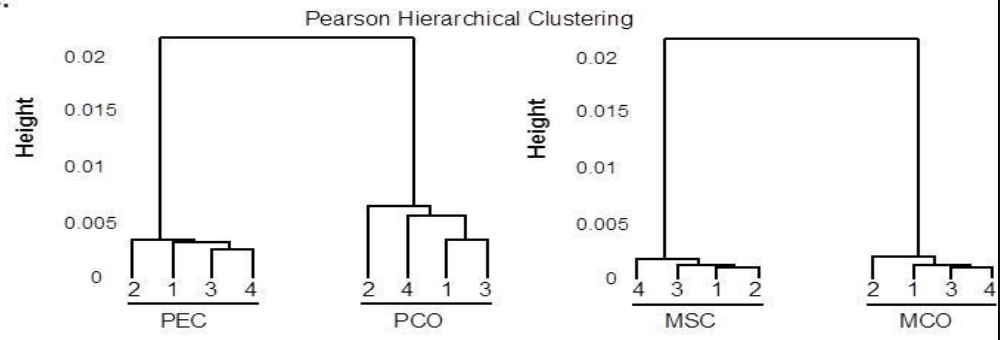
A.



B.

GENE Probes	Fold Change (PCO:PEC)
CD44	-1.09
CD44	-1.11
CD44	-1.13
CD44	1.04
CD44	-1.08
CD44	1.05

C.



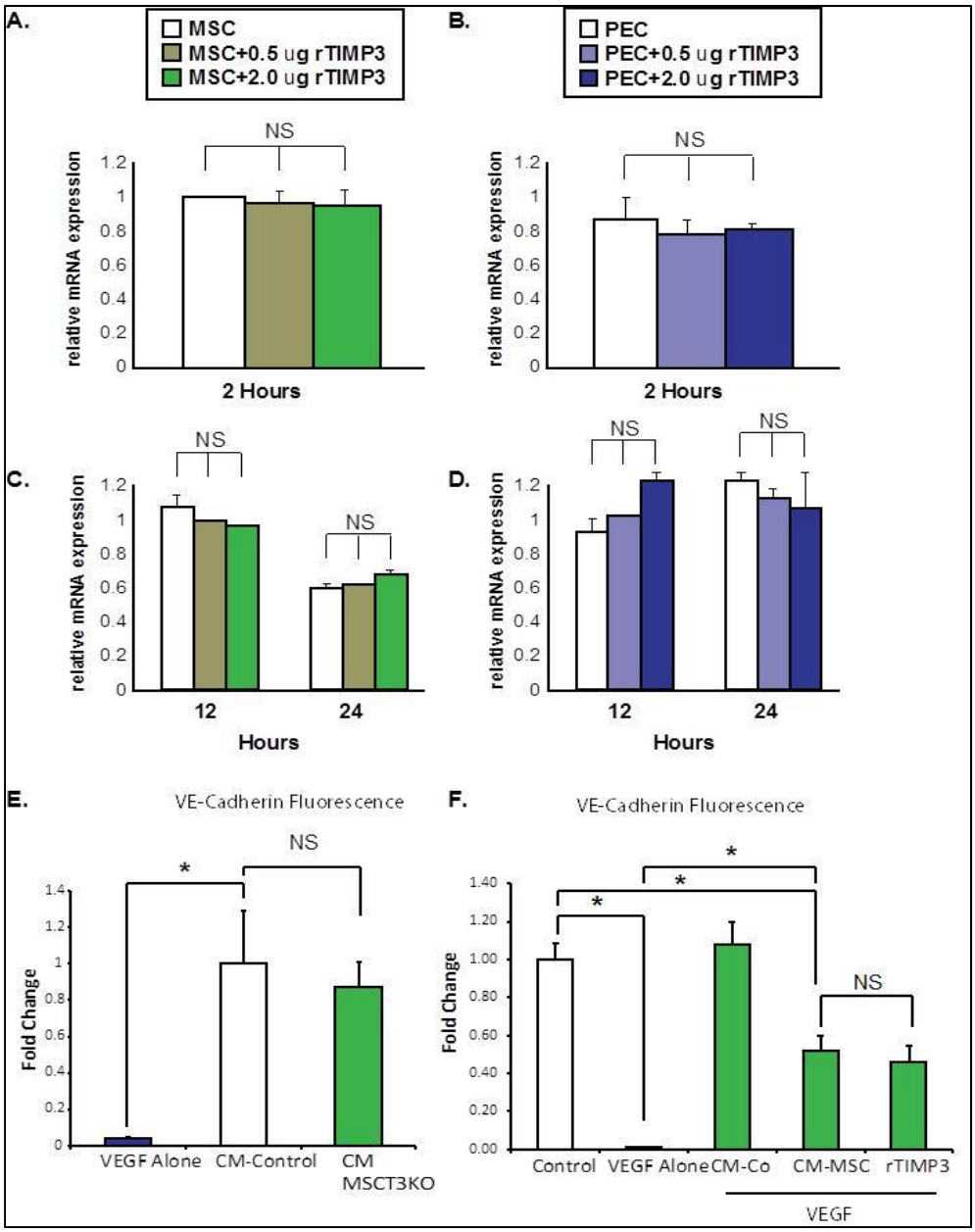
D.

	Gene	Fold Change	*P*
PCO vs. PEC	Wnt5a	19.04	1.05E-09
	TNFS-10	10.50	1.18E-05
	TGFβI	5.18	2.86E-09
	TIMP3	4.71	1.25E-07
	MMP-2	3.39	1.44E-06
	ADAM-12	2.79	2.35E-05
	TIMP1	1.86	5.21E-06
	TIMP2	1.13	1.25E-04
	Plasminogen activator,urokinase	-2.12	4.74E-05
	IL-8	-2.83	5.25E-03
	TNFS-18	-2.99	4.12E-05
	Angiopoietin 2	10.26	2.21E-07
	MMP-10	4.00	6.15E-07
	MMP-1	3.20	1.19E-05
MCO vs. MSC	ADAM-19	2.84	7.65E-06
	TGFβ-1	1.72	6.24E-08
	TIMP3	1.10	2.91E-01
	TIMP1	1.05	1.36E-01
	TIMP2	-1.32	4.03E-05
	ADAMTS-1	-2.15	1.09E-05
	FGF-5	-2.31	6.26E-07
	Angiopoietin1	-2.59	1.81E-03
	Plasminogen activator, tissue	-3.08	1.55E-06

Supplementary Figure 1. MACs separation and Affymetrix analysis of PEC-MSC co-cultures.

(S1A) Flow cytometry histogram showing that MSCs but not PECs express CD44 after MACs based separation. (S1B) Analysis of gene array data showing that PCO population is devoid of any CD44 transcripts post-MACs separation, indicating a clean separation of the co-cultured cells. (S1C) Pearson hierarchical clustering differences between PCO and PEC and MCO and MSC. (S1D) Fold-change expression in various soluble factor genes in both MSCs and PECs following direct contact. Genes were identified via the methods outlined in Figure S1A and S1B. Changes in TIMP3 expression in both the PEC and MSC groups are highlighted in red.

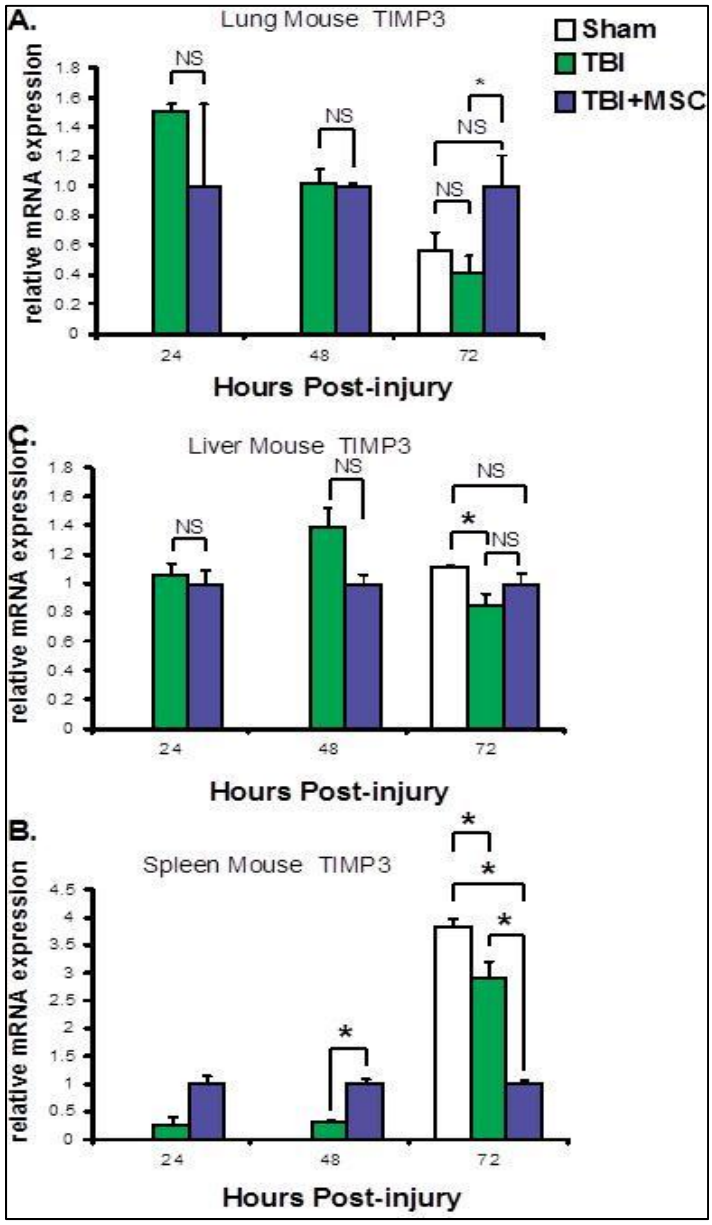
Supplementary Figure 2.



Supplementary Figure 2. Effect of rTIMP3 on TIMP3 production in MSCs or PECs in culture and Quantification of VE-Cadherin Fluorescence in PEC Cultures

qPCR analysis of TIMP3 mRNA synthesis in (S2A) MSCs and (S2B) PECs following treatment with rTIMP3 for 2 hrs. Exogenous TIMP3 does not affect production of TIMP3. qPCR analysis of rTIMP3 treatment of (S2C) MSCs and (S2D) PECs at 12 and 24 hrs demonstrates no further effect on TIMP3 production in these cells. For all experiments, data were expressed as mean ± SEM; n =2. (S2E) Cell Surface quantification of VE-Cadherin expression from Figure 2C. reveals that there is a trend to decrease (not significant though) in surface VE-Cadherin expression in PECs treated with CM from the T3KO MSC cultures.(S2F) Cell surface quantification of VE-Cadherin expression from Figure 3A. reveals that VEGF abrogates VE-Cadherin expression which is significantly preserved by CM from co-cultures (CM-Co), CM-MSC, and rTIMP3- *p<0.05, n=4/condition.

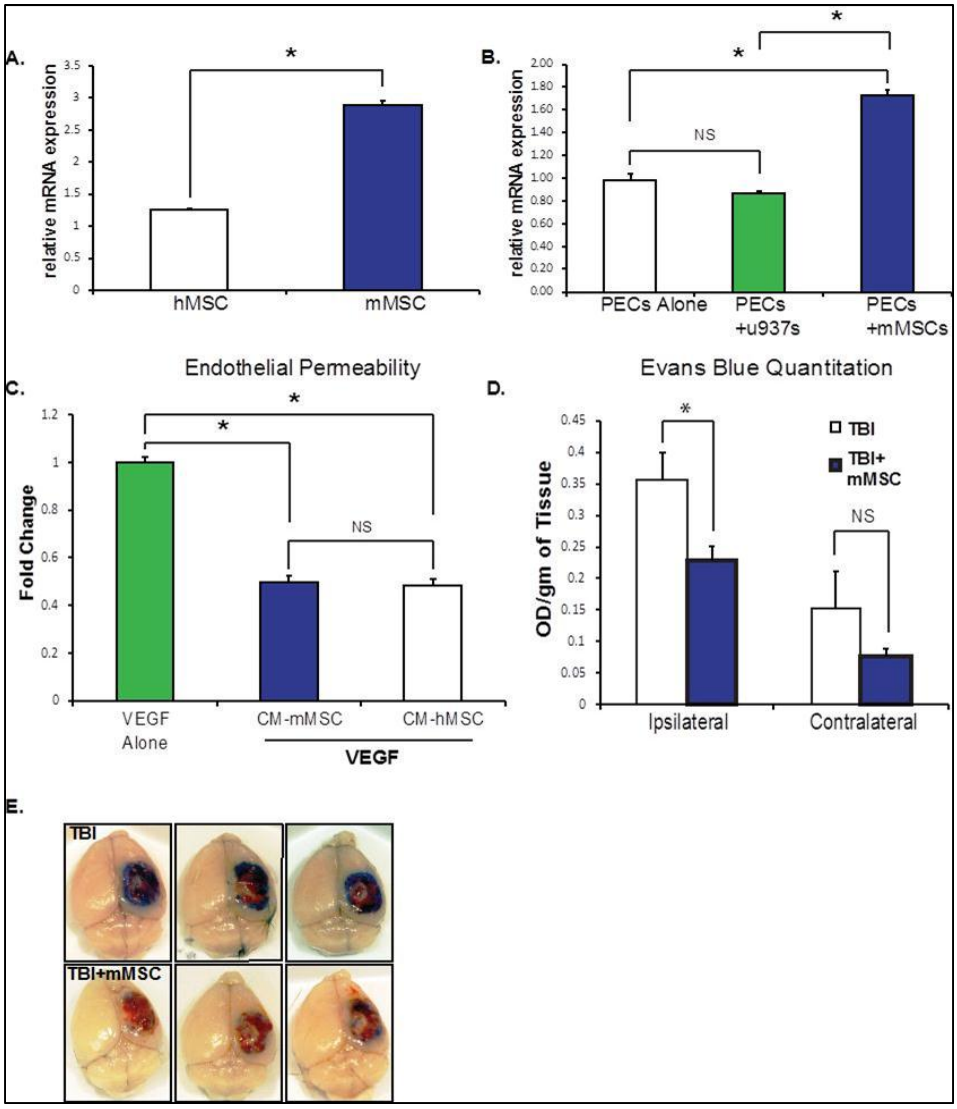
Supplementary Figure 3.



Supplementary Figure 3. qPCR analysis of TBI mouse tissue from lung, liver, and spleen in MSC treated mice.

(S3A) qPCR of murine TIMP3 levels in the lungs of sham mice (white), untreated TBI-injured mice (green), or TBI-injured mice treated with 1x10^6/dose MSCs (blue) reveals an increase (*p<0.05) in TIMP3 in MSC-treated animals at 72 hrs post-injury. (S3B) qPCR of murine TIMP3 levels in the spleen of the animals in (S3A) reveals an increase (*p<0.05) in TIMP3 in MSC-treated animals at 24 and 48 hrs post-injury. (S3C) No differences in TIMP3 expression in the liver were detected in these animals via qPCR at 24, 48, or 72 hrs post-injury. For all experiments, data were expressed as mean ± SEM; n =2-5.

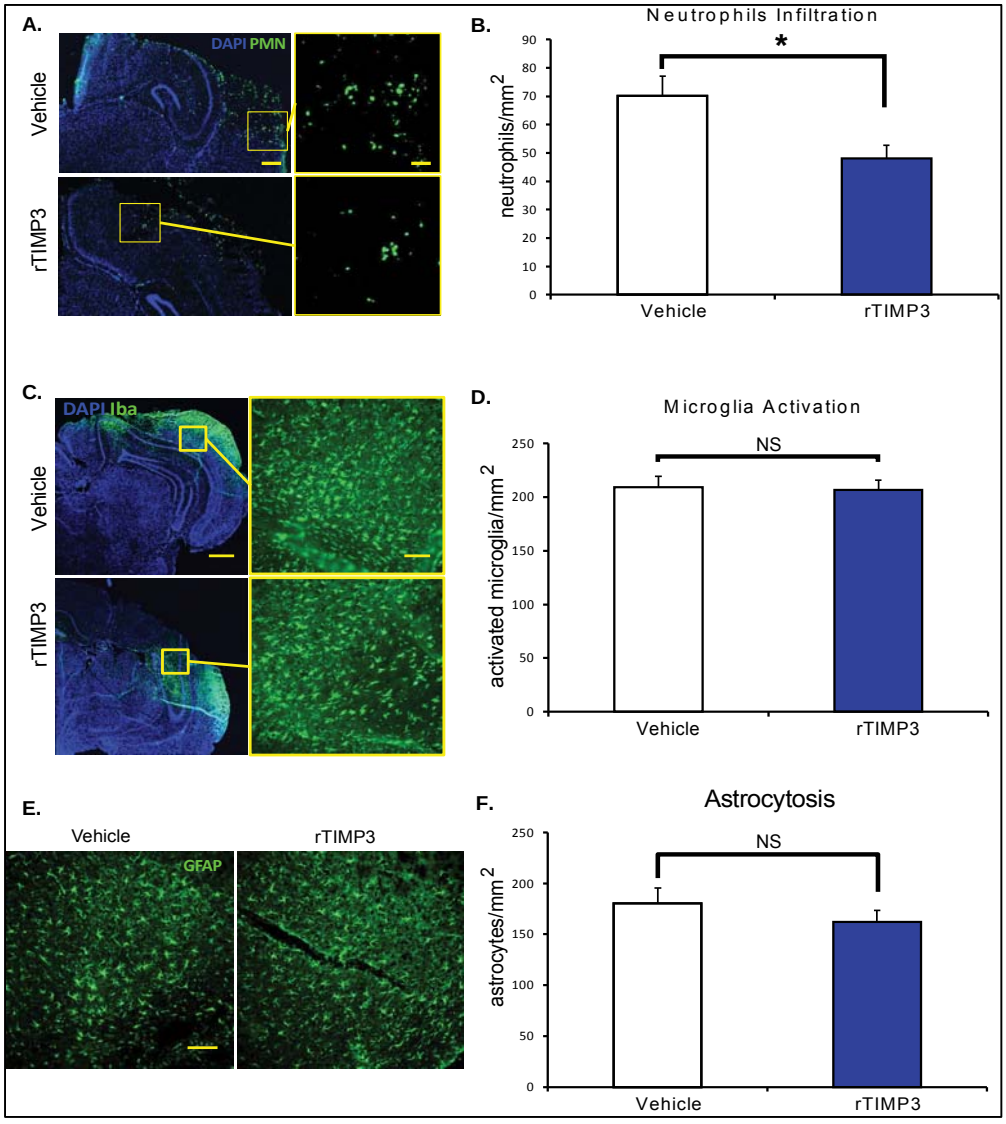
Supplementary Figure 4.



Supplementary Figure 4. TIMP3 production by murine MSCs (mMSC) and effect of mMSCs on BBB permeability induced by TBI.

(S4A) *In vitro* comparison of TIMP3 production between hMSCs (human MSCs) and mMSCs (murine MSCs) via qPCR reveals TIMP3 mRNA expression in mMSCs (*p<0.001) as found in hMSCs. Data were expressed as mean ± SEM; n = 2-6. (S4B) PECs co-cultured in contact with mMSCs show a nearly two-fold increase in TIMP3 production compared to PECs cultured alone or in contact with U937s (*p<0.001). Data were expressed as mean ± SEM; n =4. (S4C) Permeability of PEC monolayers to FITC-Dextran when cultured in the CM from mMSCs, alone (CM-mMSC) or hMSCs alone (CM-hMSC). CM-mMSC recapitulates the effect of CM-hMSC on EC permeability (*p<0.001). Data were expressed as mean ± SEM; n =4. (S4D) Quantification of Evans Blue Dye Extravasation from mice treated with vehicle (TBI) or mMSCs (TBI+mMSC) at 72hrs post-TBI. Mice treated with mMSCs demonstrated significant reduction in Evans Blue extravasation in the ipsilateral cortex compared to those treated with vehicle (*p<0.05). Data were expressed as mean ± SEM; n=9. (S4E) Representative images of the site of cortical impact injury from mice in the indicated treatment groups.

Supplementary Figure 5.



Supplementary Figure 5. Attenuated immune cell infiltration in the ipsilateral cortex of rTIMP3-treated mice following TBI.

(S5A) Neutrophils are NIMP-R14-positive (PMN) cells detected in the injured hemisphere. Images illustrate decreased neutrophil infiltration in rTIMP3 treated brains at 3 days after TBI compared to vehicle (*$p < 0.05$). (scale bar = 400 µm and 100 µm for left and right panels, respectively). (S5B) NIMP-R14 immunohistochemistry was quantified as cells/mm2. Data are expressed as mean ± SEM; n = 10. (S5C) Activated microglia are identified as Iba-positive cells. Representative images illustrate microglia activation in vehicle and rTIMP3 brains at 3 days after TBI (scale bar = 600 µm and 100 µm for left and right panels, respectively). (S5D) Iba positive cells were quantified as the cells/mm2. No significant differences were found in mice injected with rTIMP3 vs. vehicle mice. Data were expressed as mean ± SEM; n = 10. (S5E) Astrocytes were identified as GFAP-positive cells. Representative images illustrate astrocytes (GFAP positive) (scale bar = 100 µm). (S5F) GFAP was quantified as cells/mm2. No significant differences in density of astrocytes were found in TIMP3 treated mice. Data were expressed as mean ± SEM; n = 6.